ORIGINAL ARTICLE

The metallothionein and RCAS1 expression analysis in breast cancer and adjacent tissue regarding the immune cells presence and their activity

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Abstract

INTRODUCTION: The generation of proper immune response in the tumor environment seems to be essential in antitumor defense. RCAS1 expression has been shown to participate in the regulation of immune cytotoxic activity, metallothionein participates in the protection of cells against immune mediated apoptosis. Since MT and RCAS1 expression is observed within healthy tumor environment we aimed to focus on the proteins expression in tumor and its healthy adjacent tissue in invasive ductal breast cancer regarding the immune cells presence and activity.

MATERIAL AND METHODS: RCAS1, metallothionein, CD3, CD56, CD4, CD25, CD69, CD68 and CD16 antigens expression was assessed by immunohistochemistry in invasive ductal breast cancer. Tissue samples were obtained from 45 patients and were grouped according to the presence of lymph nodes metastases. Two groups were obtained: with and without lymph nodes metastases.

RESULTS: Significant differences were observed in RCAS1 and metallothionein expression in tumor and significant differences in metallothionein expression in healthy stroma regarding the presence of lymph nodes metastases. The significantly higher RCAS1 expression was noticed in tumor in comparison to stroma in patients with the presence of lymph nodes metastases. No such difference was observed in patients without the metastases. Significantly higher metallothionein expression was identified in tumor than in stroma in both groups of patients, with and without lymph nodes metastases. These changes in RCAS1 and metallothionein expression were significantly related with the changes in the number and activity of immune cells.

CONCLUSION: RCAS1 and metallothionein expression in breast cancer healthy stroma seems to be essential for the coexistence of cytotoxic immune cells and normal epithelial cells. The loss of the ability to compensate the growing cytotoxic immune response in the environment might participate in the development of tumor spread.

Introduction

The activity of cytotoxic immune cells is regulated by cancer cells and this phenomenon seems to play an important role in cancer progression. Lymphocytes accumulate in cancer vicinity but their cytotoxic activity against cancer can be inhibited by cancer itself [1]. The cancer and immune cell interaction takes place by many factors, the presence of which can be demonstrated in cancer cells and in extracellular matrix. The participation of RCAS1 (Receptor associated cancer antigen presenting on SiSo cells) in the suppression of cytotoxic immune cells in various malignant neoplasms as well as the participation of metallothionein in the protection against cancer induced apoptosis has been recently presented [1,2]. RCAS1 expression has been demonstrated in various neoplasms cell membranes including breast, pancreatic, liver, head and neck, uterine cervix, lungs, endometrium cancers [2,4,5]. Recently RCAS1 soluble form of protein has been reported to be present in blood serum and to preserve the ability to suppress immune cytotoxic activity resulting in the tumor escape from host immunological surveillance [6]. The lymphocytes apoptosis around RCAS1 positive cells has been already evaluated. Sonoda has shown that RCAS1 expression in uterine cancer cells and in metastatic cells in lymph nodes was correlated with growing number of apoptotic lymphocytes [7]. Similarly in previous reports RCAS1 expression has been associated with the number of apoptotic lymphocytes adjacent to tumor cells in lung cancer and Hodgkin's disease [4,8]. In vitro studies have also demonstrated that the culture of activated lymphocytes with RCAS1 peptides led to strong suppression of lymphocytes growth and eventually led to apoptotic cell death [9]. Reverse correlation between RCAS1 expression and cytotoxic mononuclear cells infiltration has been found in breast cancer [5]. RCAS1 expression has been linked to poor prognostic factors [10]. The participation of RCAS1 in the cancerogenesis of early breast cancer has been also demonstrated [11].

Metallothionein (MT) is a cystein rich protein and its expression is related with the regulation of cell proliferation and death [3,12–14]. MT-1 and MT-2 isoforms are ubiquitous in various human cells and cancer cells including breast cancer [15-17]. Their expression is coregulated by many factors, including: hormones, metals, and cytokines [18,19]. A relation between MT expression and growing proliferation monitored by immunohistochemical detection of Ki-67 in breast cancer cells has been shown [14]. MT expression has been also connected with reduced apoptosis in nasopharyngeal cancer and hepatocellular carcinoma [17,20]. Similarly, in breast cancer the down regulation of MT expression has been linked to the inhibition of cell growth and the development of apoptosis [21]. MT is a potential factor playing a role in determining sensitivity of tumor cells to apoptosis, and it is an inhibitor of induced apoptosis [3]. MT was observed to be a negative regulator of NF-kappa B activity [22]. MT may protect cells against p53-mediated apoptosis [23]. High MT expression in breast cancer is a negative prognostic factor [12,24]. MT expression is also linked to cytotoxic T lymphocytes suppression. Metallothionein protecting cancer cells against immune mediated apoptosis and inhibiting immune cytotoxic activity participate in tumor escape from host immunological surveillance [25].

A proper level of tumor infiltrating lymphocytes (TIL) cytotoxic activity at the tumor site is maintained by preceding TIL infiltration and accumulation and reaching the activity level. Stromal cells seem to participate in the interaction between tumor cells and immunological cells. MT and RCAS1 expression has been demonstrated not only in the tumor tissue, but also in the normal epithelium of the upper respiratory tract mucosa, bone marrow, endometrium and placenta [26–32]. The accumulation of activated immune cells within normal endometrium is a part of reproductive processes, and the MT and RCAS1 expression has been shown to participate in the regulation of immune cytotoxic activity and in the protection of endometrial cells against immune mediated apoptosis [27,32]. Since MT and RCAS1 expression is found within healthy epithelium of tumor vicinity we aimed to focus on the proteins expression in tumor and its healthy adjacent tissue in ductal breast cancer regarding the immune cells presence and activity.

Material and methods

Group of patients

In all cases patient's informed consent was received. The approval for the research program from the Ethical Committee of the Jagiellonian University in Krakow: KBET/379/13/2003 was also obtained. The patients in this study were randomly selected. Surgically removed material was evaluated to determine histological type and metastases of the lymph nodes using histological methods in the Department of Patomorphology of the Jagiellonian University. The clear surgical margin was defined as 1 cm^2 area of tumor adjacent tissue macroscopically and histological free of neoplasmatic texture. The surgical resection line was macroscopically and histological free from cancer texture.

Tissue samples

The study group consisted of 45 patients with breast cancer. All women underwent mastectomy with axillary lymphadenectomy in 1st General Surgery Department of Jagiellonian University. The women age ranged between 33–78 years (mean age 59 years). In each case invasive carcinoma was a dominant component. In all cases surgical material was obtained after modified radical amputation of the breast according to Patey's method with simultaneous removal of axillary lymph nodes. Surgical material was fixed in 10% buffered formalin. Then tissue was embedded in paraffin and stained with hematoxylin and eosin. Each specimen was inspected to specify tumor size and a number of lymph nodes obtained for study. Microscopic examination was performed to identify

Table 1. The scale used for evaluation of metallothionein and RCAS1 expression.

Antigen	Immunoreactivity			
	0	+1	+2	+3
RCAS1	No reactivity	Weak (when observed any, also granular in paranuclear region) cytoplasmic staining pattern in up to 10% of positive cells	Marked cytoplasmic (sometimes together with membranous) staining in 11–30% of the cells	High expression – more than 30% of positive cells
Metallothionein	Lack of any positivity	Weak staining in less than 5% of the cells	Moderate – various staining intensity but in <50% of the cells,	Strong – staining of more than 50% of the cells.

histological type and grade of invasive carcinoma, the presence of vessel invasion and tumor metastases to the lymph nodes. Histological grades of invasive carcinoma were diagnosed according to Bloom and Richardson classification modified by Elston and Ellis, recommended by the National Coordinating Group for Breast Pathology [34].

Immunohistochemistry

Immunohistochemical analysis was performed in the Patomorphology Department of the Jagiellonian University. Five-micrometer slides from each case were stained to visualize expression of RCAS1, MT and CD16-, CD25-, CD69-, CD4-, CD3-, CD56-positive cells (mainly lymphocytes) as well as CD68+ cells, it means macrophages. In all cases immunohistochemistry was performed applying Envision method using Dako Autostainer. The following antibodies were applied: mouse monoclonal antibody Anti- RCAS1 (Medical and Biological Laboratories, Nakaku Nagoya, Japan in DAKO Antibody Diluent with Background Reducing Components-DAKO, Denmark, dilution 1:1 000), monoclonal mouse antibody ImmunOTM (MP Biomedicals, Inc., clone 1A12 in dilution 1:1 000), CD56 (NCAM; NCL-CD56-504, Novocastra) in dilution 1:100, CD69 (NCL-CD69, Novocastra) in dilution 1:25, CD25 (Interleukin-2 Receptor, NCL-CD25-305, Novocastra) in dilution 1:25, CD16 (NCL-CD16, Novocastra) in dilution 1:40, CD68 (Klone PG-M, Dako) in dilution 1:50, according the manufacturer's instructions, CD3 (NCL-CD3p, Novocastra) in dilution 1:100 and CD4 (Klone 1F6)in dilution 1:150. Visualization of reaction products was performed using AEC (3-amino-9-ethyl-carbazole) as a chromogen (AEC Substrate Chromogen ready-to-use, DAKO, Denmark) for 10 minutes at room temperature. Sections were counterstained with hematoxylin and mounted in glycergel. As a positive control a tonsil specimen was taken for RCAS1 and for metallothionein it was a breast cancer specimen. All stainings were performed with the same procedure but with the omission of the primary antibody as a negative control.

RCAS1 expression was evaluated in entire slides in cancer cells and in glandular epithelium lining the ducts and acini of healthy stroma, considering the intensity of the colour reaction and percentage of cells. MT expression was evaluated in cancer cells, glandular epithelium lining the ducts and acini of healthy stroma and in stromal myoepihelial cells. The percentage of cells and degree of color reaction in MT-positive cells was analyzed. The scales used for estimation of the above both markers staining is shown in Table 1.

The immune cells were calculated in an entire specimen, in the tumor and in region around tumor and an average cell number per 2 hpf (high power field, objective magnification × 40) was calculated. Variable scales were used to evaluate semiquantitatively an amount of

Table 2. The scale used for evaluation of CD25, CD56,CD3, CD4, CD68, CD69, and CD16 antigens expression.

Antigen	Immunoreactivity				
	0	1+	2+	3+	4+
CD 25	Lack of positive cells	Single positive cells	1–5 positive cells/1hpf	More than 5 positive cells/1hpf	-
CD 56	Lack of positive cells	-	-	-	-
CD 4	Lack of positive cells	-	-	-	-
CD 69	Lack of positive cells	Single positive cells	1–5 positive cells/1hpf	More than 5 positive cells/1hpf	-
CD 3	Lack of positive cells	1–5 positive cells/1hpf	6–10 cells/1hpf	11–20 positive cells/1hpf	More than 20 positive cells/1hpf
CD68	Lack of positive cells	1–5 positive cells/1hpf	6–10 cells/1hpf	11–20 positive cells/1hpf	More than 20 positive cells/1hpf
CD16	Lack of positive cells	1–5 positive cells/1hpf	6–10 cells/1hpf	11–20 positive cells/1hpf	More than 20 positive cells/1hpf

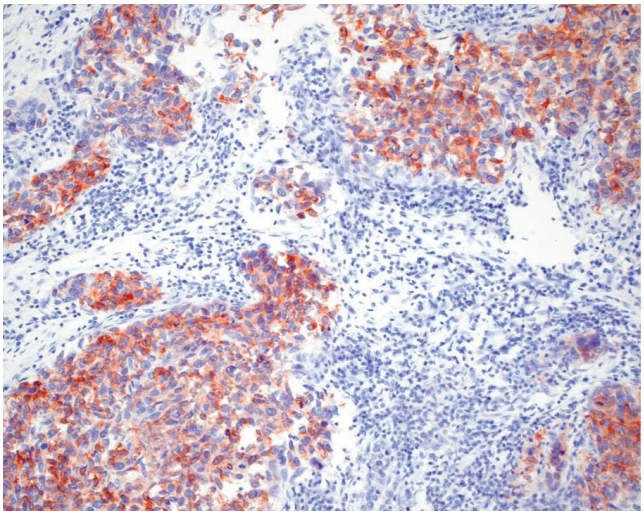

Figure 1. Strong RCAS1 expression (3+) in cancer cells in patient with the presence of lymph nodes metastases.

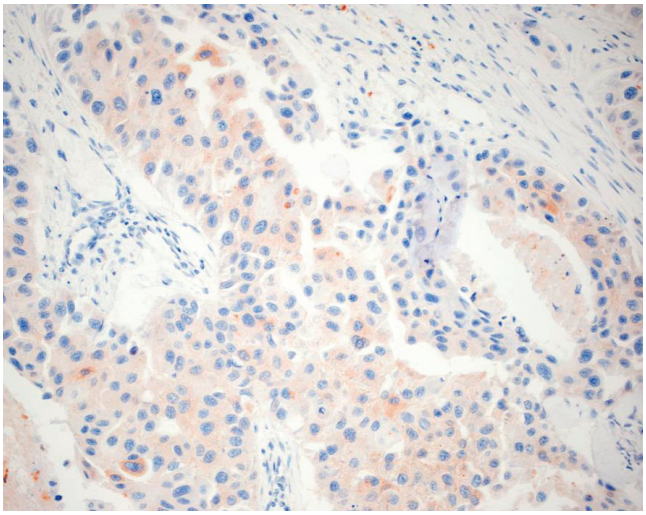

Figure 2. Weak RCAS1 expression (1+) in cancer cells in patient without the presence of lymph nodes metastases.

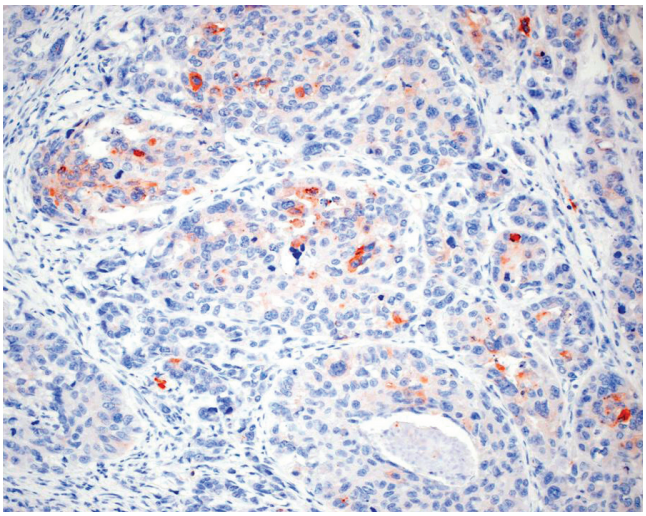

Figure 3. Strong MT immunoreactivity (3+) in cancer cells in patient with the presence of lymph nodes metastases..

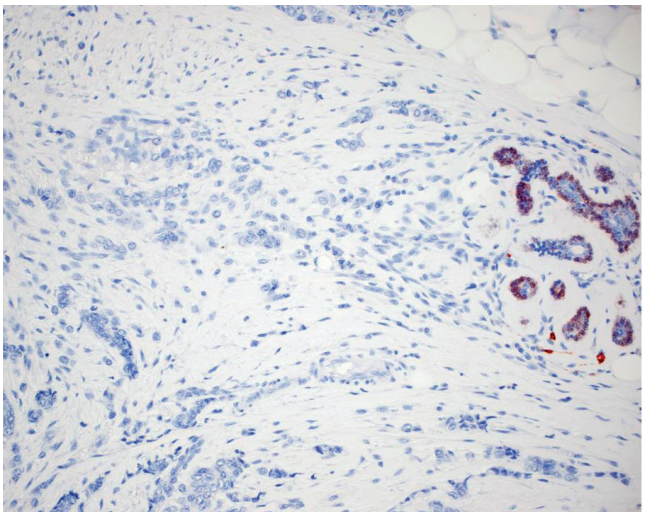

Figure 4. The lack of MT immunoreactivity in cancer cells, stroma and normal glandular cells in patient without the presence of lymph nodes metastases..

the cells, depending of their general number in the specimen, what is summarized in the Table 2.

The evaluation of immunohistochemical reactions was performed independently by two histopathologists (L. R-S. and K. G.).

Statistical analysis

The distribution of variables in the study groups of women checked with the use of the Shapiro-Wilk test showed that all of them were different from normal. Therefore, nonparametric testing was employed. Statistical significance between the groups was determined by the Kruskal-Wallis analysis of variance (ANOVA) test. The Mann-Whitney U test was then used as applicable. The Spearman rank test was used to evaluate interclass correlation coefficients. All calculations were carried out with the use of STATISTICA software v. 6 (StatSoft, USA, 2001).

Results

RCAS1 and MT immunoreactivity was analyzed in invasive ductal breast cancer and its healthy stroma. RCAS1 expression was identified in cancer nests and in glands of healthy stroma.

MT expression was identified in cancer, glands of healthy stroma and in stromal myoepithelial cells.

The patients were divided into two main groups according to the presence of lymph nodes metastases. The patients with the presence of lymph nodes metastases had statistically significantly lower breast cancer cells' differentiation according to Bloom scale than the patients without metastases (p=0.04)

Table 3. The evaluation of RCAS1 immunoreactivity with respect to the presence of lymph nodes metastases.

Variables	N status	RCAS1 - Immunoreactivity			
		0	+1	+2	+3
RCAS1 tumor expression	N0 (n=22)	40 (9)	45 (10)	5 (1)	10 (2)
	N1 (n=23)	21 (5)	34 (8)	34 (8)	11 (2)
RCAS1 stromal expression	N0 (n=22)	63 (14)	27 (6)	5 (1)	5 (1)
	N1 (n=23)	60 (14)	30 (7)	5 (1)	5 (1)

Table 4. The analysis of metallothionein expression with respect to the presence of lymph nodes metastases.

Variables	N status	MT - Immunoreactivity			
		0	+1	+2	+3
MT cancer expression	N0 (n=22)	31 (7)	22 (5)	36 (8)	11 (2)
	N1 (n=22)	22 (5)	27 (6)	36 (8)	15 (3)
MT expression in the healthy glands adjacent to cancer	N0 (n=22)	11 (2)	17 (4)	36 (8)	36 (8)
	N1 (n=22)	-	5 (1)	27 (6)	68 (15)
MT myoepithelial expression	N0 (n=22)	73 (16)	22 (5)	5 (1)	-
	N1 (n=22)	41 (9)	44 (10)	15 (3)	-

Table 5. The comparison of CD3, CD25, CD69, CD16, CD68 antigens expression in tumor and its adjacent healthy stroma.

Tumor antigen expression	Stromal antigen expression	p-value
CD3	CD3	0.02
CD25	CD25	0.3
CD69	CD69	0.9
CD16	CD16	0.02
CD68	CD68	0.7

Table 6. The evaluation of RCAS1 and MT immunoreactivity and the presence and activity of immune cells.

Proteins	Lymphocyte antigen	p-value
RCAS1 stroma	CD3 stroma	0.07
RCAS1 stroma	CD25 stroma	0.02
RCAS1 tumor	CD16 tumor	0.02
MT tumor	CD3 tumor	0.047
MT stroma	CD25 stroma	0.034

The analysis was performed by Kruskal-Wallis analysis of variance (ANOVA) test.

Analysis of RCAS1 immunoreactivity

Statistically significantly higher RCAS1 expression was observed in the group of tumors with the presence of lymph nodes metastases than in the group without metastases ($p<0.001$). No differences in RCAS1 expression were observed in cancer healthy stroma regarding the presence of lymph nodes metastases. RCAS1 expression in tumor was statistically significantly higher than in stroma in patients with the presence of lymph nodes metastases ($p=0.0002$), no such difference was observed in patients without the presence of lymph nodes metastases.

The presence of lymph nodes metastases correlated with RCAS1 expression ($r=0.57$, $p<0.001$). A correlation was also observed between RCAS1 expression in tumor and the tumor grade/cells differentiation according to Bloom scale ($r=0.48$, $p<0.001$). We compared RCAS1 expression and the tumor differentiation using Kruskal-Wallis analysis of variance (ANOVA) test ($p=0.0045$). RCAS1 expression in Bloom 2 and 3 grades were on comparable levels and were statistically significantly higher than in Bloom 1 ($p<0.01$ in both cases).

Analysis of MT immunoreactivity

MT expression was statistically significantly higher in tumors with the presence of lymph nodes metastases than in tumors without the presence of lymph nodes metastases ($p<0.01$). Similarly healthy glands adjacent to cancer represented statistically significantly higher MT expression in cases with the presence of lymph nodes metastases in comparison to cases without metastases ($p=0.01$). Tumor stroma represented statistically significantly higher MT immunoreactivity in tumors with the presence of lymph nodes metastases when compared to tumors without metastases ($p<0.01$). MT expression was statistically significantly higher in tumor than in stroma in both groups of patients, with and without the presence of lymph nodes metastases (respectively in both cases $p<0.001$).

MT expression correlated with the presence of lymph nodes metastases in tumor tissue ($r=0.46$, $p<0.01$), healthy stromal glands ($r=0.36$, $p=0.01$) and in myoepithelial cells of healthy stroma ($r=0.41$, $p<0.01$).

The highest level of MT expression was identified within tumor tissue, stromal glands and myoepithelial cells of healthy stroma in Bloom 2 tumors, while the lowest level of MT was found in Bloom 1 tumors, however the differences were not statistically significant.

Immune cells

The presence of CD3 positive cells was observed in both, tumor and its adjacent healthy tissue. No CD56 positive cells and no CD4 positive cells were observed neither in tumor nor in healthy stroma. The activity of immune cells was evaluated by identification of CD25 and CD69 antigens expression in both, tumor and its adjacent healthy stroma. Additionally CD16 and CD68 antigens expression were evaluated.

The number of CD3 positive lymphocytes was statistically significantly higher in tumor adjacent healthy stroma in the group of patients without the presence of lymph nodes metastases in comparison to the group with metastases (p=0.01). The number of CD3 positive TIL was higher in the group without the presence of metastases in comparison to the group without metastases, but the difference was not statistically significant. Similarly, the number of CD25 positive cells was higher in the group without metastases in both tumor tissue and tumor adjacent healthy stroma in patients without the presence of metastases when compared to the group with metastases. The expression of CD69 antigen was on comparable level in both groups, with and without the presence of metastases. CD3 expression correlated statistically significantly with CD25 expression (r=0.45, p<0.01), and with CD69 (r=0.52, p<0.001) in tumor.

The presence of immune cells and their activity was analyzed in our study regarding the MT immunoreactivity and RCAS1 immunoreactivity changes within tumor and tumor adjacent healthy tissue. The obtained results are presented below in Table 6.

Statistically significant correlation was observed between RCAS1 tumor expression and CD68 antigen expression (0.29, p=0.046) and CD16 antigen expression (0.36, p=0.02). Statistically significant correlation was also observed between MT stromal glands expression and CD25 antigen expression (r=0.27, p=0.06) and between MT stromal myoepithelial cells expression and CD25 antigen expression (r=0.04, p<0.01).

Discussion

Statistically significant differences were observed in RCAS1 and metallothionein expression in tumor and statistically significant differences in metallothionein expression in healthy stroma with respect to the presence of lymph nodes metastases. These changes in RCAS1 and metallothionein expression were significantly related with the changes in the number and activity of immune cells.

RCAS1 expression in breast cancer has already been considered however its expression in tumor adjacent healthy stroma has not been analyzed yet. RCAS1 is a protein responsible for the tumor escape from host immunological surveillance. A relation between EBAG9 (oestrogen receptor-binding fragment associated gene 9) gene overexpression the product of which has later been identified to be identical with RCAS1 plays an important role in the development and progression of breast cancer in the early stage of its growth [11]. In 2001 Suzuki reported a significant association between ER-alpha (estrogen receptor alpha) status and RCAS1 expression [5] and confirmed this finding in his further study in 2004 [35]. The relation between ER-alpha and RCAS1 has been statistically significant in ERR-alpha (estrogen related receptor alfa) positive tumors. ERR possibly modulates the expression of ERE containing estrogen responsive genes. ERR expression is an independent negative prognostic factor, correlates with the recurrence of the disease and adverse clinical outcome of the ductal breast carcinoma, has been found to be significantly associated with the presence of lymph nodes metastases [35]. In our study RCAS1 correlated statistically significantly with the presence of lymph nodes metastases. Although such a correlation has not been identified in the study of Suzuki [5]. Additionally, on the contrary to Suzuki report, RCAS1 expression in tumor in our study was statistically significantly correlated with tumor grade (0.48, p<0.01). The patients with the presence of lymph nodes metastases had statistically significantly more frequently poorly differentiated tumors than the patients without the presence of lymph nodes metastases. The statistically significantly higher RCAS1 expression was noticed in tumor in comparison to stroma in patients with the presence of lymph nodes metastases. No such difference was observed in patients without the metastases. High RCAS1 expression in tumor with the presence of metastases was associated with the lower number of CD3 positive TIL and lower CD25 expression, this finding confirms Suzuki report [35], in which RCAS1 expression was inversely correlated with the number of CD3 positive cells. The concomitant to tumor dissemination RCAS1 growth in tumor with its decrease in stroma may result from the disturbance of regulatory mechanisms in the tumor environment what might condition the further progression.

The differences between types of immune cells and their activity found between breast cancer and its healthy stroma are interesting. Statistically significant differences were observed in the number of CD3 positive cells and the CD16 antigen expression between tumor and its healthy adjacent tissue. The interaction between tumor and immune system is still not well understood. The ability of tumor cells to regulate the immune cells activity seems to be essential for tumor escape from host immunological surveillance. It has been already reported that tumor cells polarize an antitumor defense into Th2 response [1]. This phenomenon leads to the down regulation of perforin expression within TIL or to blockade of its cytotoxicity trough stimulation of CD94/NKG2A heterodimer expression on NK cells and even on other CTLs [1]. Predominant Th2 anticancer response may lead to down regulation of the cytotoxic potential of TIL, what results in the altered cytokines pattern in tumor milieu [36]. Cancer cell may alter the functional composition of anti-tumor effect cells through the decrease of CD25 antigen expression [37]. In our study RCAS1 stromal immunoreactivity changes were statistically significantly associated with CD3 and CD25 antigen stromal expression. The alteration of the number of TIL subpopulation may be realized by mutual affection of various cytokines in tumor milieu. The growth or domination of Th2 polarity is related with the raise of IL-10 in cancer milieu and leads to the modulation of IL-12 action. From one side IL-12 is essential for the activation of NK cells, from the other side IL-12 in the presence of IL-10 may stimulate CD94/NKG2A receptor on NK and CTLs [38,39]. This complex regulation of immune response show the simultaneous inhibitory and activating mechanisms ap-

pearing in tumor environment. The observed decreased expression of the IL-2 receptor in TIL in tissue adjacent to the tumor in breast and cervical cancers has been interpreted by Sheu as a result of existence of TIL selective suppression phenomenon [37]. This can easily be explained by selective suppression phenomenon using RCAS1 expression. The biological role of the selective suppression phenomenon is not clearly understood and seems to result from the physiological necessity of the coexistence of activated lymphocytes together with adjacent host cells. Our results and these presented above indicate that this coexistence is maintained by RCAS1 stromal expression. The presence of lymph nodes metastases is the evidence of tumor dissemination and indicates that the selective suppression phenomenon is disrupted in tumor environment.

Activated lymphocytes accumulate in tumor adjacent healthy stromal tissue in breast cancer. From one side these cells (cancer cells) posses an ability to regulate the immune cells activity and from the other side posses also mechanisms protecting them from unwanted apoptosis. Metallothionein expression promotes cells proliferation and protects cells from apoptosis [3]. Mutual interaction between MT2A gene activity and ECRG2 has been reported. The product of ECRG2 gene is responsible for the inhibition of proliferation and induction of apoptosis in esophageal cancer. On the contrary to ECRG2, MT promotes cells proliferation and protects cells from apoptosis [13]. The homeostasis of cancer cell seems to be maintained through the balance between these two genes activity/expression. MT plays an important role in determining the sensitivity of tumor cells to apoptosis [40]. The reduction of MT expression in MCF-7 human breast cancer cell line resulted in inhibition of cell growth and in the initiating of spontaneous apoptosis [21]. MT seems to regulate the process of apoptosis by the influence on the Zn^{2+} intracellular level which proper concentration is essential for the caspases: 3, 8 and 9 activity [41]. MT immunoreactivity represented both cytoplasmic and nuclear pattern of staining in breast cancer cells [12,14,42]. Similarly to these findings we observed MT immunoreactivity in both cytoplasm and nuclei. MT expression in cytoplasm is thought to be related with protection against cytotoxicity while its nuclear expression is related with the protection against genotoxicity [23,43]. MT expression in breast cancer is a factor of poor prognosis [24,44]. No relationship has been found between MT isoforms expression and the expression of estrogen receptor in breast cancer [45]. MT expression seems to participate in tumorogenesis of estrogen receptors negative ductal breast cancers [41]. A relationship between MT mRNA and the tumor grade has been observed in breast cancer, G3 tumors were typified by MT-2A mRNA overexpression [14]. MT immunoreactivity in our study was observed to be in both tumor and healthy stroma at the highest level in G2 tumors and at the lowest level in G1 tumors, however the differences were not statistically significant. The differences may result from the alterations in mRNA expression and immunohistochemistry analysis of intra-cellular distribution [15]. In our study differences in MT1/2 reactivity between the epithelium and stromal glands as well as in myoepithelial cells were observed. No such differences were observed in MT2A gene expression, it has been suggested to result from gene post-transcriptional regulation rather than from gene expression alterations and this might explain the relationship between MT1/2 expression raise and cancer disease progress [15]. Metallothionein expression in breast epithelial cells typifies the malignant ductal epithelium [46], while MT expression in myoepithelial cells has also been observed in healthy cells [47]. In our previous study MT expression was determined in breast cancer stroma and was raising statistically significantly in tumors with the presence of lymph nodes metastases, therefore we suggested that MT expression might result from the interaction between spreading tumor cells and the cells from healthy adjacent stroma [48]. Statistically significantly higher MT expression has been revealed in tumor tissue, healthy stromal glands and in myoepithelial stromal cells in cases with the presence of lymph nodes metastases in this study in comparison of cases without lymph nodes metastases.

MT expression may raise also in response to various acute phase cytokine factors including: interleukins (Il-1, Il-6), tumor necrosis factor (TNF-alfa) and INF-gamma [18,49–51]. Metallothionein seems to participate in the interaction between tumor cells and immune cells and enables the tumor cells to evade the host defense immune response [25]. Young J revealed that MT expression can contribute to dramatic decrease of cytolytic activity of immune cells and plays an important role in the suppression of anti tumor immunity [25]. Statistically significant correlation between stromal MT immunoreactivity and CD25 expression on immune cells was identified in our study. Similar observation reported Youn in an in vitro study, demonstrating high MT expression with high CD25 expression independently of suppression of cytolytic CTLs activity. Therefore Youn suggested that the participation of MT in tumor escape from the host immunological surveillance may be related with its suppression of immune cell's response and induction of cancer cell's proliferation. MT expression may be secondary to accumulating immune cells and the growing cytotoxic activity. In our study a statistically significant association was identified between MT immunoreactivity changes and the presence of CD3 positive cells in tumor and between MT stromal immunoreactivity and CD25 antigen stromal expression. MT expression in tumor and its healthy adjacent tissue seems to be secondary to the activity of immune cells infiltrating tumor and protects MT expressing cells against immune mediated apoptosis. This phenomenon is necessary also for the assuring of tissue integrity during growing cytotoxic response against tumor cells and the loss of this ability may lead to tumor spread. The tumor environment is typified by the MT overexpression which may be the demonstration of physiological local processes regulation destruction in

cases of the presence of lymph nodes metastases and the tumor dissemination occurs.

Conclusions

The selective suppression of activated immune cells and the ability to acquire resistance to immune mediated apoptosis are essential factors enabling the interaction between activated immune cells with cells of tumor adjacent environment. RCAS1 and metallothionein expression in breast cancer and its healthy stroma seem to be essential for this coexistence. The loss of the ability to compensate the growing cytotoxic immune response in the environment might participate in the development of tumor spread.

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